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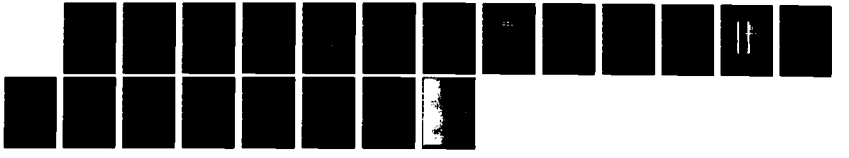

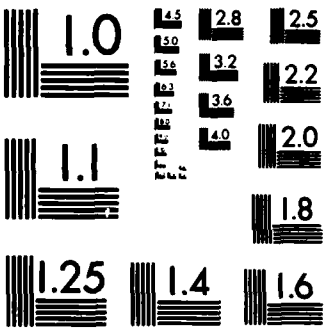

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Report Number 3

Replication of Japanese Encephalitis Virus

Final Report

by

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Dept. of Microbiology

July 1980 - September 1981

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ABSTRACT

Gene expression in the flavivirus Japanese encephalitis virus (JEV) was studied by three different approaches.

Virus-specific RNA in infected cells was radiolabeled in the presence of actinomycin D, and analyzed by sucrose gradient sedimentation and agarose gel electrophoresis. In addition to the 40S genome RNA and a probable replicative intermediate, two small single-stranded species, designated 15S RNA and 12S RNA, were observed. They were shown by oligonucleotide mapping to be subsets of the 40S RNA and different from each other. Their kinetics of synthesis suggested that that they functioned in virus replication, probably as subgenomic mRNAs, and were not merely breakdown products of 40S RNA.

Virus-specified proteins were separated from host cell proteins by radioimmune precipitation and analyzed by polyacrylamide gel electrophoresis and fluorography. A total of 13 different polypeptides were seen, with molecular weights ranging from 98,000 to 10,000. Their total molecular weight exceeded the coding capacity of the virus genome.

A library of 15 temperature-sensitive (ts) mutants of JEV were induced by growth of virus in the presence of mutagens. All had efficiency of plating at 41 C as compared to 33 C of 10^{-2} or less. Some failed to make virus-specific proteins or RNA at 41C. Genetic complementation tests indicated that the mutants represented at least five different gene functions.

Table of Contents

I. RNA from virus infected cells and virions

1. Virion RNA. Vero cells were infected with JEV at multiplicities of 1-10 and grown in the presence of $^{32}PO_4^{-2}$ from 24-72 hr PI. Virions were purified from medium by polyethylene glycol precipitation and equilibrium-followed by rate zonal-gradient sedimentation. Labeled RNA extracted from virions sedimented as a single peak at 40S on sucrose gradients. Two-dimensional electrophoresis of RNase T1-resistant oligonucleotides demonstrated at least 30 characteristic large oligonucleotides (Fig. 1)

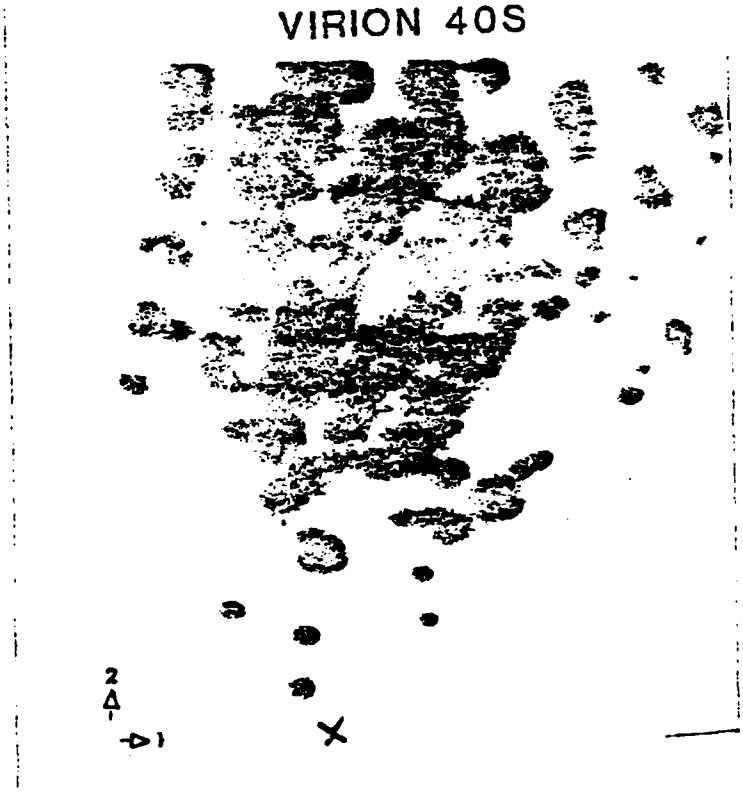

Fig. 1. ^{32}P-labeled genome (40S) RNA was isolated from purified virions by SDS-phenol extraction and sucrose gradient sedimentation. It was digested to completion with RNase T1 and resulting oligonucleotides were separated by 2-dimensional electrophoresis in polyacrylamide gels and detected by autoradiography. Arrows show direction of electrophoresis and X's show position of dye markers.

Intracellular 40S RNA had an identical fingerprint to viron RNA. However, 36S intracellular RNA lacked 3 of the large oligonucleotides from 40S RNA, suggesting that it might be a defective form rather than a conformational variant.

2. Intracellular RNA. As reported previously, a number of different size classes of virus-specific RNA are found in flavivirus-infected cells. On sucrose gradients these sediment at 40S, 36S, 22S, and 15S. Agarose gel electrophoresis of native or denatured intracellular RNA suggests that the 22S peak is a double-stranded replicative intermediate, that the other species are single-stranded, and that the 15S species consists of two different size classes, designated 12S and 15S (Fig. 2).

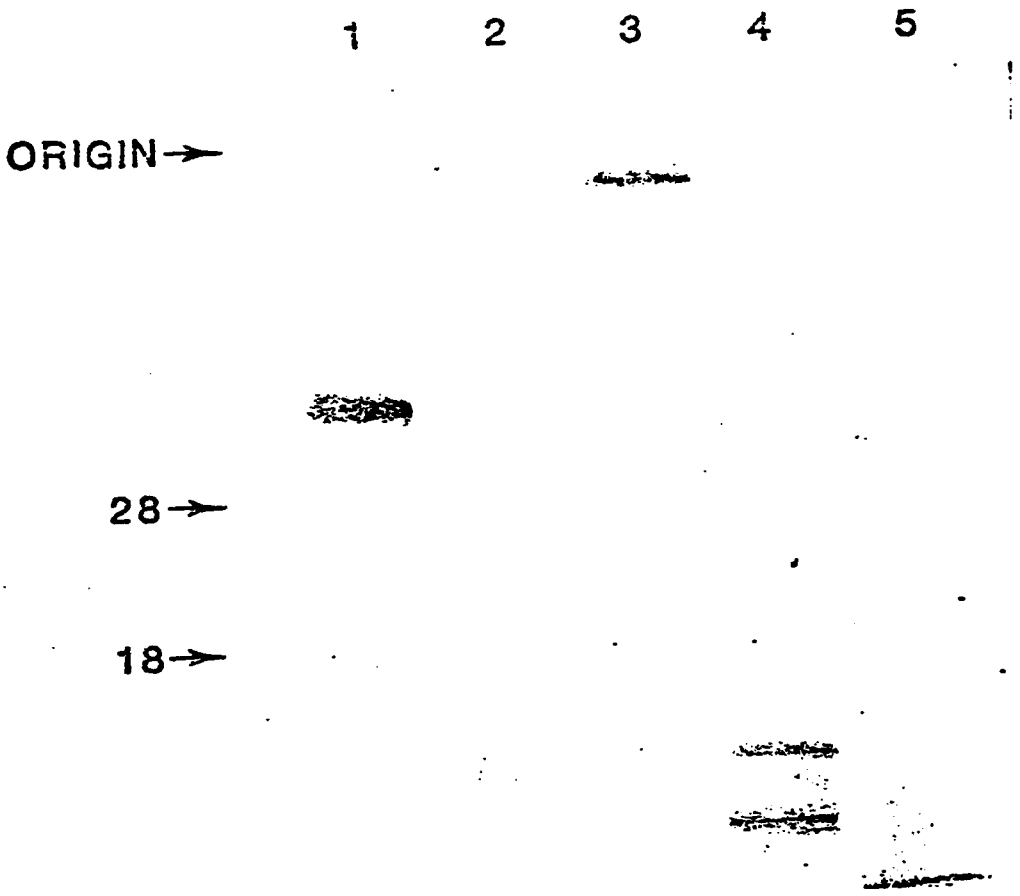

Fig. 2. Sucrose gradient fractionated RNA was precipitated and electrophoresed in a 1.2% agarose slab gel. Ribosomal 28S and 18S RNAs were located by staining with ethidium bromide. RNA species corresponding to sucrose gradient peaks which sedimented at 40S (lane 1), 28S (lane 2), 22S (lane 3), 12-15S (lane 4) and 4S (lane 5) were located by autoradiography or staining.

Labeling intracellular RNA for 4-hr pulses throughout the growth cycle followed by gel electrophoresis or gradient sedimentation of the products demonstrated that the 12-15S RNA species are detectable as early as 3 hr PI and throughout infection, whereas 40S RNA only becomes detectable at 12-16 hr PI (Fig. 3).

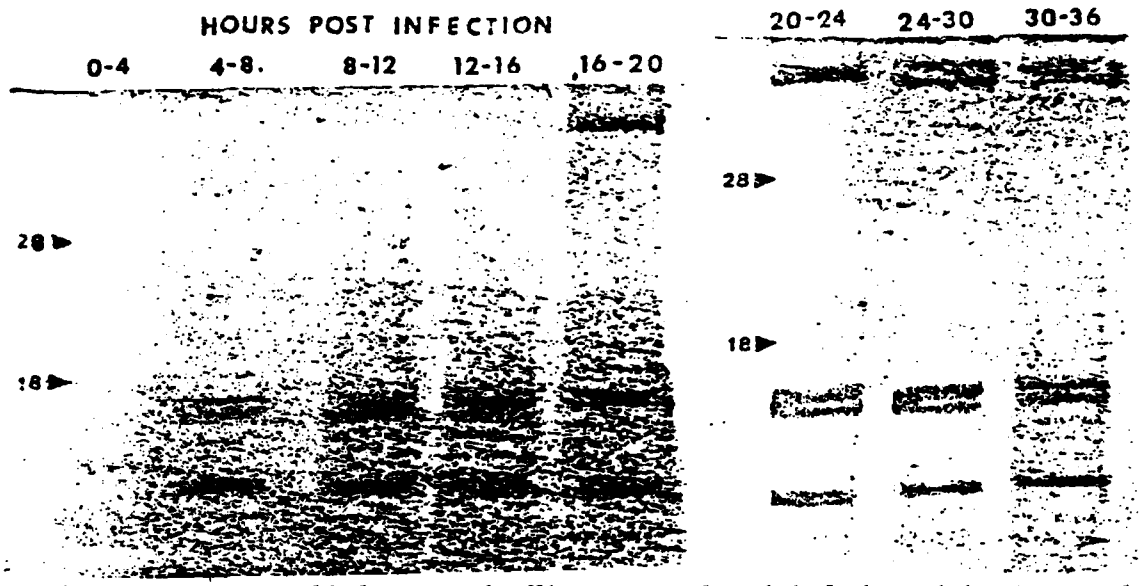

Fig. 3. Intracellular viral RNAs were pulse-labeled at 4-hr intervals in the presence of actinomycin D. RNAs were extracted and electrophoresed in 1.4% agarose slab gels. The 12-15 RNAs were evident from 4-36 hr, 40S from 16-36 hr PI.

The 36S RNA occurs late in infection in highly variable amounts. These data suggest that whereas 36S may be a defective variant of virion RNA, the 12S and 15S species are synthesized independently and are not breakdown products of 40S RNA.

Maps of RNase T1-resistant oligonucleotides indicate that the 12S and 15S species each share sequences with the 40S RNA, though not with each other, and that each has a small number of unique oligonucleotides (Fig. 4).

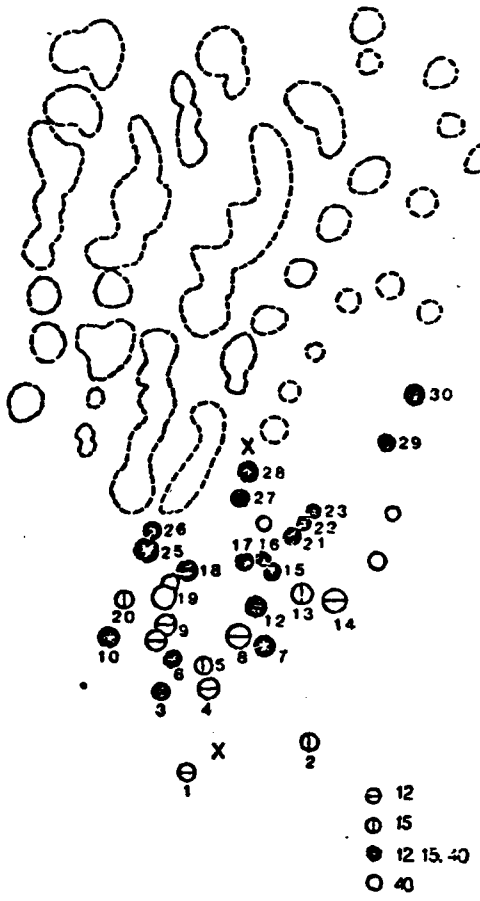

Fig. 4. Individual 12S, 15S, and 40S RNA species were extracted from agarose gels, digested with RNase T1, and electrophoresed separately and in combination. Schematic shows relationship of 30 largest oligonucleotides to the individual oligonucleotides of each RNA species.

Since the virion RNA is infectious, it is considered an mRNA, even though it lacks poly (A). These results suggest that the 12-15S RNAs have positive polarity and are also probably virus-specific mRNAs. The unique oligonucleotides in each species may result from splicing during their synthesis or from imprecision in their start and stop sites. Further evidence that the 12-15 S RNAs are subgenomic species was obtained by synthesizing complementary DNA to 40S RNA with reverse transcriptase and using the cDNA as a hybridization probe for 15S and 12S RNAs. The above results have been submitted as manuscript 1. in Bibliography.

3. Cellular site of RNA synthesis. Synthesis of all virus-specific RNA leading to virus production is unaffected by exposure to the transcription inhibitor actinomycin D and the DNA synthesis inhibitor mitomycin C, as shown in Table 1.

Table 1

Inhibitor	Concentration (μg/ml)	Time of treatment hr PI	Yield of JEV at 24 hr (PFU/ml)
None			2.7×10^6
Act D	5	1-3	1.3×10^6
Act D	5	3-5	3.6×10^6
Act D	5	5-7	1.7×10^6
Act D	5	12-24	2.0×10^6
Mit C	10	-8 to 0	4.1×10^6
Mit C	50	-8 to 0	4.3×10^6

Autoradiography and cell fractionation reveal that all virus-specific RNA synthesis takes place in the cytoplasm of host cells. The virus must therefore utilize its own RNA-dependent RNA polymerase. These results have been submitted as manuscript 2 in Bibliography.

II. Virus-specified proteins

1. Radioimmune precipitation. Since JEV does not shut off host cell protein synthesis, virus-specific proteins must be identified against a cellular background. Use of actinomycin D, cycloheximide reverse, and high-salt reverse aided somewhat in inhibiting cell protein synthesis, and 6-7 virus-specified proteins were identified in infected cells (see last annual report). However, use of specific antisera to precipitate viral proteins from infected cell lysates allowed identification of 13 viral proteins, ranging in molecular weight from 98,000 to 10,000. Hyperimmune mouse ascitic fluid containing antibodies to JEV-infected cell proteins was obtained from Dr. Nick Karabatsos, CDC, Ft. Collins. Antiserum was incubated with ^{35}S-methionine-labeled lysates from both infected and uninfected cells. Immune complexes were precipitated by binding to Staphylococcus A protein. Precipitated proteins were analyzed by polyacrylamide gel electrophoresis (PAGE) and fluorography. Uninfected cell lysates contained no polypeptides which reacted with antiserum. Molecular weights of immune-precipitated proteins in infected cells were 98K, 95K, 81K,

60K, 56K, 51K, 43K, 41K, 29K, 20K, 15K, 11K, and 10K. Their total molecular weight was >600,000, a figure which is beyond the coding capacity of the 40S genome. By comparison to virion structural proteins, the intracellular proteins with mol wts of 60K and 56K were identified with the virion glycoprotein, probably as glycosylation isomers. The other two structural proteins did not have intracellular equivalents (Fig. 5).

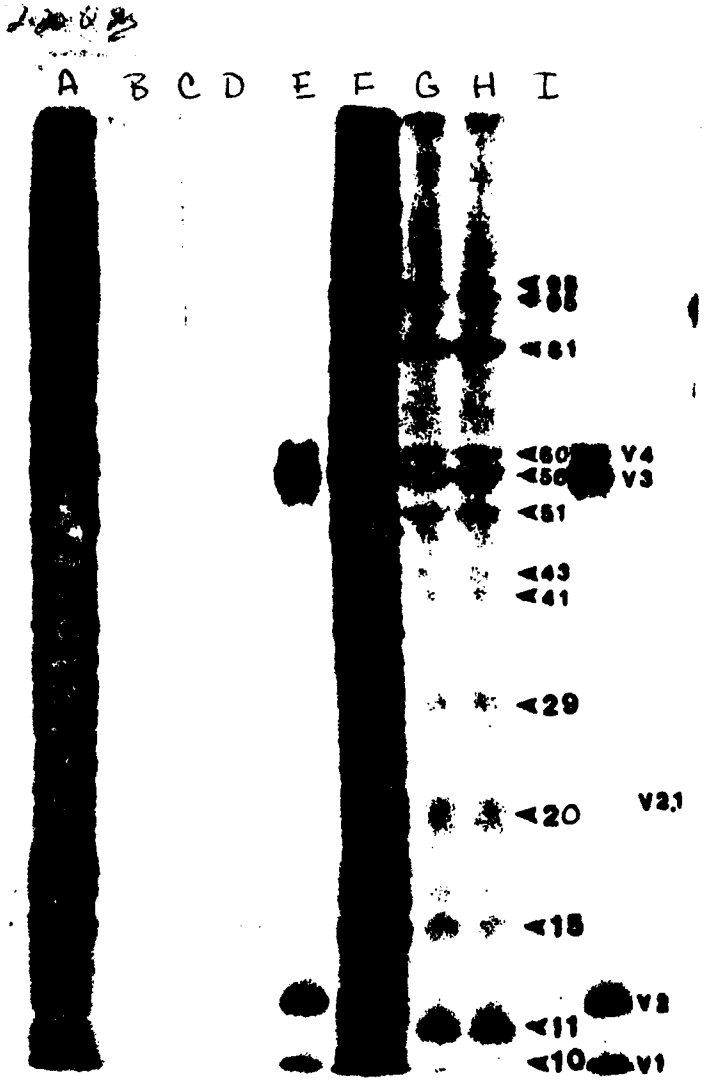

Fig. 5. JEV-infected and uninfected SW13 cells were labeled with ^{35}S-methionine from 24-25 hr PI. Cells were lysed with a buffer containing NP40 and Triton X-100, and reacted with hyperimmune mouse ascitic fluid, and precipitated with Staphylococcus aureus cells. Reaction mixtures were boiled with SDS-2-mercapto-ethanol and subjected to PAGE and fluorography. Lane A = untreated, uninfected cell lysate; lanes B, C, D = uninfected cell lysate reacted with hyperimmune ascitic fluid; lane E = virion proteins; lane F = untreated infected cell lysate; lanes G, H = immune precipitated infected cell lysate; lane I virion proteins.

Traces of two polypeptides with molecular weights >100,000 were seen on some immunoprecipitate gels and were accentuated after treatment

of infected cells with the protease inhibitor Trasylol. These data suggest that processing and possible cleavage of structural and probably nonstructural JEV proteins occurs during virus replication. Continuing work involving peptide analysis and development of monoclonal antibodies to JEV proteins will help to elucidate polypeptide interrelationships. A manuscript is in preparation regarding the above protein work.

2. Cell-free translation. Both 40S and 15S RNAs isolated from infected cells by sucrose gradient sedimentation have been shown to stimulate incorporation of amino acids into polypeptides. When analyzed by PAGE, these peptides do not have mobilities directly comparable to those from infected cells. However, a number of different peptides are precipitated by hyperimmune ascitic fluid, signifying their virus specificity. It is probable that the products in the cell-free translation system are either prematurely terminated or unprocessed virus-specific proteins. Further work with the system will determine conditions for completion and peptide mapping will assist in identification of products.

III. Isolation and characterization of temperature-sensitive (ts) mutants of JEV

A library of fifteen ts mutants of Japanese encephalitis virus has been developed. The mutants were generated by growth of wild type virus (WT) in the presence of the base analogs 5-azacytidine (AC) and 5-fluorouracil (FU). The resulting stock of virus was plaque assayed at 33 C and as many well isolated plaques as possible were picked using a sterile pasteur pipet. The virus was eluted into 0.5 ml medium and the resultant stocks screened for the ability to kill PK 15 cells at 33 C, the permissive

temperature, but not at 41 C, the nonpermissive temperature. Those clones of virus which appeared TS after screening were plaque assayed at the permissive temperature and plaques picked, as previously described, for purification. After three plaque purifications, stocks of each clone were prepared. The stocks were then plaque assayed at both the permissive and nonpermissive temperatures. Those clones not showing at least a 100 fold reduction in growth at the nonpermissive temperature relative to the permissive temperature, i.e., efficiency of plating equal to or less than 10^{-2}, were discarded. The remaining clones which did demonstrate the ts characteristic were used for biochemical and genetic studies.

The mutants were then characterized as to their protein and RNA pehnotypes, i.e., production of virus-specific proteins and RNA, respectively, at the nonpermissive temperature. The percent reversion of the mutant stock to wild type virus as well as the percent leak (those virus particles which grow at the nonpermissive temperature yet retain their ts characteristic) was determined. Table 2 presents the data from the mutants so far tested.

Some of the mutants were tested for the ability to complement the ts lesion in another mutant by comparison of the virus yield (assayed at 33 C) from a doubly infected culture grown at 41 C relative to the sum of the yield from cultures singly infected with each mutant at 33 C. Complementation indices of two or greater are considered significant and underscored in Table 4.

Table 4

Mutants	68	92	96	128	90	161	204
68							
92	0.2						
96	2.5	2.9					
128	0.3	1.1	1.0				
90	44	2.1	35	0.1			
161	8.9	1.0	10	0.3	29		
204	46	1.0	7.4	0.2	4.3	18	

Several observations should be made from Table 4. Mutant 128 does not complement any other mutant and mutant 92 complements very poorly. Both of these mutants, however, have high leak percentages as seen in Table 2. With the exception of these two mutants, five complementation groups are defined indicating at least five gene functions. It should also be noted that eight mutants remain to be characterized as to complementation. Additionally, no recombination was observed in these studies.

Conclusions

Our results open new possibilities for the mechanism of replication of JEV. Subgenomic mRNAs, synthesized early in replication, may code for non-structural proteins required for RNA replication or virion assembly. The demonstration of virus-specific polypeptides with a greater coding capacity than the genome suggests protein cleavage and processing. The library of ts mutants capable of undergoing complementation may now be exploited to determine total number of gene products and mechanisms by which they are synthesized.

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Degrees to be completed.

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P. Scott Eastman	Ph.D.	1982.

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